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14. ABSTRACT

The objective of this study is to develop an effective prophylactic therapy against an analog of mustard gas, 2-chloroethylethyl sulfide (CEES). The therapy for CEES-induced skin damage will be based on the topical application of antioxidant liposomes. We will use EpiDerm cultured human skin tissues as well as cultured keratinocytes as working models. HD/CEES-induced pathophysiology involves oxidative stress. Liposomes, containing both water- and lipid-soluble antioxidants, are expected to be an effective antidote/therapeutic product to protect US military and civilians from a chemical warfare agent such as HD. The therapeutic efficacy, as well as the chemical and physical stability of various antioxidant liposome formulations, will be examined to determine the optimal preparation for future testing in animal models.

15. SUBJECT TERMS
skin, CEES, toxicity, liposomes, antioxidants, vitamin E, protection, keratinocytes

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ABBREVIATIONS:

ALA, α-Lipoic acid
AT, α-tocopherol
CEES, half mustard or 2-chloroethyl-ethyl sulfide
CAM, calcein AM
Car-DCFH DA, carboxy-dichloroflurescin diaceatate
DEVD-AFC, peptido (DEVD)-7-Amino-4-trifluoromethylcoumarin
DMSO, dimethyl sulfoxide, a solvent
GSH, reduced glutathione and an antioxidant
GT, γ-tocopherol
HD, sulfur mustard or bis-2-(chloroethyl) sulfide
IL-1β, interleukin-1 beta
LPS, lipopolysaccaride
MNNG, N-methyl-N'-nitro-N-nitrosoguanidine
MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAC, N-acetyl-L-cysteine
NFκB, nuclear factor kappa-B
PI, propidium iodide
PMA, phorbol myristate acetate
ROS, reactive oxygen species
TBHP, tert-butyl hydroperoxide
TNF-α, tumor necrosis factor-alpha

INTRODUCTION:

Sulfur Mustard (HD): The sulfur mustard (bis-2-(chloroethyl) sulfide) could effectively be used to produce casualties in the battlefield and to force opposing troops to wear full protective equipment thereby slowing down the tempo of military operations. It could also be used with devastating results against civilian targets, and such use of HD by S. Hussein's military forces in Iraq has been well documented. The extensive and slow healing skin lesions following exposure to HD would also place a heavy burden on the medical services of military and public health organizations.

Effects of HD on human skin: A characteristic of sulfur mustard exposure is the occurrence of a symptom free period of some hours post exposure. The duration of this period and the severity of the subsequent lesions are dependent upon the mode of exposure, the environmental temperature and individual to individual variations. In the first hour after exposure to mustard gas vapor or liquid no signs or symptoms are usually produced, but nausea, vomiting and eye smarting have been occasionally reported. Post-exposure (up to 24 hours) skin inflammation ensues followed by lesion formation and blistering.

Treatment of HD: There is no antidote or effective treatment for mustard gas intoxication.

Mechanism of CEES/HD-induced skin damage: Oxidative stress is an important mechanism for HD induced skin injury. HD and its analog 2-chloroethylethyl sulfide (CEES) induce alkylation of DNA, and rapid oxidation of intracellular proteins and lipids. CEES and HD are known also to react with the major intracellular antioxidant GSH; depleting it with a subsequent loss of protection against reactive oxygen species (ROS) and an activation of inflammatory responses.

Oxidative stress is an important mechanism for HD induced skin injury. HD and its analog 2-chloroethylethyl sulfide (CEES) induce alkylation of DNA, and rapid oxidation of intracellular proteins and lipids. CEES and HD are known also to react with the major intracellular

antioxidant GSH; depleting it with a subsequent loss of protection against reactive oxygen species (ROS) and an activation of inflammatory responses.

Considerable evidence suggests that HD toxicity is associated with an increased generation of damaging free radical production and promote apoptosis. As detailed below, we have found that oxidative stress and pro-inflammatory cytokines play a key role in the toxicity of CEES. The PI's laboratory is part of a DOD funded group termed the "Advanced Medical Countermeasure Consortium". This group is systematically evaluating the overall hypothesis that oxidative stress, pro-inflammatory agents, and apoptotic cell signaling are the key factors in the toxicity of mustard gas. In recent years, there has been an enormous expansion of findings on the molecular mechanism of inflammatory responses and its relationship to oxidative stress [1-4].
We are currently taking advantage of this rich wealth of background information to help define the molecular links between inflammatory agents, oxidative stress and mustard gas toxicity. We have found that macrophages exposed to CEES have a decreased level of intracellular GSH, which is even further diminished in the presence of LPS [5]. Pretreatment of the macrophages with N-acetyl cysteine (NAC) protects against the loss of intracellular GSH. NAC serves to promote the synthesis of GSH and, this is the likely mechanism for the protective effect of NAC against CEES/HD toxicity as reported by the PI's laboratory and other researchers.

The HD-induced depletion of GSH together with protein and lipid oxidation has far reaching consequences that have not been previously appreciated. HD-induced pathophysiology may occur in large part due to a disruption of redox homeostasis. Redox homeostasis is dependent upon the balance between oxidants and antioxidants. Redox sensitive gene expression is determined by the redox status of the cell. Signal transduction events induced by endogenous stimuli alter the redox state of the cell. GSH depletion influences a variety of cellular signaling process, such as activation and phosphorylation of stress kinases (JNK, p38, PI-3K) via sensitive cysteine-rich domains; activation of sphingomyelinase ceramide pathway, and activation of AP-1 and NFkB, with subsequent gene transcription [6, 7]. GSH levels are inversely related to the activity of NFkB [9]. NFkB regulates many genes involved in inflammation such as: inducible nitric oxide sythase (iNOS), proinflammatory cytokines, Il-1, TNF-alpha, interleukin 6 (IL-6),

chemokine, IL-8, E-selectin, vascular cell adhesion molecule 1 (ICAM-1), and granulocyte-macrophage colony stimulating factor (GM-CSF) [6, 8].

Arroyo et al. found a dose dependent increase in TNF- alpha, IL-6, IL1 beta in SM treated human keratinocyte cells [9]. Signal transduction has also been demonstrated for CEES, wherein TNF-alpha, sphingomyelinase levels, caspase 3, 8, and 9 were all elevated [10]. Stone et al. [5] have found that inflammatory cytokines exacerbate the toxicity of CEES. In proliferating cells GSH levels are rapidly depleted by TNF-alpha. It is postulated that oxidative stress induced by CEES is further amplified by the loss of GSH and inflammatory cytokine production, thus exacerbating CEES-induced pathophysiology.

Rationale for the use of Liposomal Antioxidants: One way of reversing or preventing the ROS-induced cellular injury is via topical application of antioxidants. Vitamin E (tocopherols and tocotrienols), GSH, N-acetylcysteine (NAC), and lipoic acid are very effective antioxidants. Their antioxidative potential and importance in skin pathophysiology had been tested previously in a large number of investigations [2, 11-14]. However, the delivery of antioxidants to skin remains problematic in some cases. The intact skin allows the passage of small lipophilic substances but, in most cases, efficiently retards the diffusion of water-soluble molecules.

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes are unique in their ability to simultaneously deliver both water-soluble (in their aqueous inner space) and lipid-soluble antioxidants (in the phospholipid bilayer) to cells and tissues. They represent an ideal drug delivery system that enhances penetration of the active ingredient into the skin, localizes the drug at the site of action, and reduces percutaneous absorption.
Stone et al. [15] have reviewed the use of antioxidant liposomes in the general area of free radical biology and medicine as well as the relevant application of this technology to weapons of mass destruction.

The term “antioxidant liposome” is relatively new and refers to liposomes containing lipid soluble chemical antioxidants, water-soluble chemical antioxidants, enzymatic antioxidants, or combinations of these various antioxidants. Antioxidant liposomes hold reatment of many diseases and conditions in which oxidative stress plays a prominent role. Several studies have clearly indicated that the liposomal antioxidant formulations compared to that of the free non-encapsulated antioxidants exert a far superior protective effect against oxidative stress-induced tissue injuries.

Military Significance: The overall objective of this study is to develop an effective prophylactic therapy against CEES-induced skin damage (analogous to HD effect) based on the topical application of antioxidant liposomes. Our preliminary data suggest that antioxidant liposomes are very effective in preventing CEES toxicity to stimulated macrophages. This study will determine potential effectiveness of various liposome formulations in ameliorating the CEES-induced skin injury. The successful outcome from this research will comprise a treatment that will reduce or prevent casualties in the battlefield thus preserving combat effectiveness. Furthermore, the timely administration of this treatment regimen will also avert the devastating results of CEES against civilians that might be exposed to CEES during a terrorist attack. In this investigation, we hope to optimize the combination of antioxidants in the liposomes to achieve the maximum therapeutic effect. In addition, we will address practical issues with regards to the large scale preparation and storage of antioxidant liposomes with long-term physical, chemical and pharmacological stability.

BODY:

Originally, we proposed using EpiDerm cultured human skin tissues as a working model to study CEES cytotoxicity and protective effects of antioxidant liposomes. However, EpiDerm tissues are expensive, and in order to save resources, we have chosen cultured normal human epidermal keratinocytes (NHEK) purchased from Cambrex to use in our first series of experiments. In addition, we have used cultured 1106 KERTr human keratinocytes (ATCC) and immortalized human keratinocytes HaCaT purchased from Cell-Lines-Service (Germany), which are easy to

culture and allow unlimited number of passages, whereas NHEK spontaneously transform after 5-7 passages (a major and costly limitation). These issues were also discussed and recommended by USAMRICD scientist Dr. R. Ray who specializing in studies of HD toxicity mechanisms. We plan to collect the bulk of preliminary results with CEES (cell viability, apoptosis, cytokine release, oxidative stress parameters) and to optimize antioxidant liposome formulations using cultured human keratinocytes, and then perform final experiments with the EpiDerm tissues. As discussed previously, this strategy has proven useful since we have already optimized the technique for delivering maximally toxic levels of CEES to cultured skin cells by use of dimethyl sulfoxide (DMSO).

Task 1: In these initial experiments we will characterize the toxicity of CEES to the keratinocytes or EpiDerm model as a function of CEES dose and exposure time. We will also determine: (1) how apoptosis contributes to CEES induced toxicity by measuring both capase-3 activity and DNA fragmentation; (2) if immuno-stimulators (LPS, PMA) and pro-inflammatory cytokines (TNF-α, IL-1β) increase CEES toxicity to human keratinocytes/EpiDerm tissues. It is postulated that apoptosis is the major cell death mechanism at low doses of CEES or HD whereas necrosis is the dominant mechanism at higher concentrations of CEES or HD and long-time exposures. We further anticipate that immuno-stimulators such as pro-inflammatory cytokines, LPS and PMA will enhance CEES toxicity to human keratinocytes.

Task 2: Similar to HD, CEES induces oxidative stress in the skin cells resulting in ROS generation, DNA damaging, protein and lipid oxidation, depletion of intracellular glutathione and vitamin E (in particular, α-tocopherol and γ-tocopherol). We will determine the influence of CEES on various oxidative stress parameters using cultured human keratinocytes and, later, the EpiDerm model. Particular emphasis will be placed on intracellular ROS monitoring, GSH/GSSG ratio, tocopherol depletion/tocopheryl quinone formation, and protein oxidation.

Task 3: We will examine the pharmacokinetics of the antioxidant liposomes following their topical application to the human keratinocytes or EpiDerm tissues. Human skin cells will be

treated with antioxidant liposomes containing both water-soluble (GSH, NAC, α-lipoic acid (ALA)) and lipid-soluble antioxidants (α- and γ-tocopherols). Cellular redox status (protein oxidation, GSH/GSSG ratio, tocopherol/tocopheryl quinone ratio) will be measured as a function of dose and treatment time. We will optimize the liposome formulations in order to achieve optimal enrichment of the cells with the antioxidants.

Task 4: We anticipate that liposome-encapsulated antioxidants will be more effective than free non-encapsulated antioxidants in preventing CEES toxicity in human skin cells. We will test the effectiveness of topical application of the optimal antioxidant liposome formulations (which will be determined in Task 3) against the CEES-induced skin damage. Cultured human keratinocytes and EpiDerm system will be utilized to reveal most protective antioxidant liposome formulations.

Task 5: We will explore the possibility of manufacturing antioxidant liposomes in large scale industrial quantities. We will further optimize antioxidant liposome formulations in order to enhance their long-term physical, chemical and therapeutic stability. The most protective antioxidant liposomes (which will be determined in Task 4) will be further characterized for the physical and chemical stability as a function of storage time and conditions. Optimal storage conditions will be found out. The liposome therapeutic effectiveness will be also monitored during long-term storage under various conditions.

Progress during fist year (summary):

Task 1: We have investigated cytotoxic effect of CEES on cultured human keratinocytes. We found that NHEK cells were much more resistant to CEES induced inhibition of cell growth than other cell lines tested with a similar experimental design. Fully grown NHEK cells slowly undergo terminal differentiation and, therefore, more resistant to genotoxic agents, such as CEES or HD, than actively proliferating cell cultures. Later, we substituted them for immortalized

HaCaT human keratinocyte, which proliferate continuously, and, unlike NHEK cells, establish a higher susceptibility to CEES toxicity than NHEK.

In order to determine if an addition of immuno-stimulators (LPS, PMA) and pro-inflammatory cytokines (TNF-α, IL-1β) increase CEES toxicity to NHEK keratinocytes, we performed further experiments with NHEK cells exposed to various levels of CEES. None of these immuno-stimulators enhanced toxicity of CEES as measured by the MTT assay. The results sufficiently differ with our previous results obtained with murine macrophages simultaneously exposed to CEES and LPS [5].

Task 2: We have done preliminary experiments in order to monitor oxidative stress parameters in CEES/ethanol treated NHEK keratinocytes. We have found that CEES induces high levels of superoxide production in NHEK cells. Superoxide levels were measured using specific fluorescent dye – dihydroethidium (HEt). Intracellular oxidation of HEt (measured as fluorescence of the product) reflects superoxide generation in cytoplasm. Interestingly, the production superoxide anion in NHEK cells appears to increase in a similar manner as CEES induced toxicity. The data confirm that oxidative stress, and ROS generation in particular, is an important factor in CEES induced cell death.

Task 4: We have initialized experiments with four different types of antioxidant liposomes in order to test their protective abilities against CEES toxicity in normal human keratinocytes. We found that liposomes containing both GSH and alpha-tocopherol were protective against CEES and, also, increased cell proliferation without CEES. Most protective effect was documented with NAC and NAC/ alpha-tocopherol (NAC/T) containing liposomes. In NHEK keratinocytes NAC/T-liposomes were not only protective, but also stimulated proliferation of NHEK cells both in the presence and in the absence of CEES. Blank liposomes (vesicles of similar size

distribution composed only with phospholipids and cholesterol, but not containing antioxidants) did not show any protective effect, as was expected.

Task 5: In order to optimize antioxidant liposome preparation we have obtained and installed the instrumentation required to manufacture antioxidant liposomes in large quantities (up to 20 L per day) and to characterize their particle size distribution. M-110 L Laboratory Microfluidizer® Processor, which allows us to produce liposomes at a rate of 270 ml/min at 18,000 PSI, was used for liposome preparation. This device was chosen since this technique can be scaled-up to industrial levels without changing liposomes properties/characteristics, which is important for future FDA considerations. Model 380 Nicomp particle size analyzer was used for liposome particle size distribution measurement. The PI and Dr. Hongsong Yang have been trained by the Field Representative of Microfluidics Company on the use of M-110L Laboratory Microfluidizer® Processor and the Model 380 Nicomp particle size analyzer.

We have optimized conditions for liposome preparation and long-term storage. The large unilamellar liposomal (LUV) formulations containing various antioxidants (GSH, NAC, α-tocopherol) have been optimized based upon: (1) best particle size distribution using a dynamic light scattering Model 380 Nicomp particle analyzer; (2) stable liposome antioxidant (e.g., vitamin E) content; and (3) absence of cytotoxicity in human skin cells. In addition, liposomes containing encapsulated water-soluble antioxidants, such as GSH and NAC, were tested on their chemical stability (oxidation) and physical stability (leakiness).

Progress during second year:

Specific Task 1:

In this year we continue experiments with cultured human keratinocytes. Previously, we reported a number of experiments with normal human NHEK cells. However, fully grown NHEK cells

slowly undergo terminal differentiation and, therefore, more resistant to genotoxic agents, such as CEES or HD, than actively proliferating cell cultures. Also, NHEK cells are expensive as every experiment requires freshly isolated batch. In order to overcome such limitations we have substituted these cells for a continuously proliferating immortalized HaCaT human cell line (Cell-Lines-Service, Germany). Indeed, we found that immortalized HaCaT human keratinocyte, unlike NHEK cells, establish a higher susceptibility to CEES toxicity. Therefore, HaCaT keratinocytes were used for the majority of our second year experiments.

Application technique is very important for the experiments with CEES or HD as both these toxic agents undergo rapid hydrolysis in aqueous solutions [16, 17]. CEES/HD can be applied without any vehicle by direct mixing in the medium immediately prior the application. Although the latter method of CEES application is widely used, it allows hydrolysis at some extent. It takes a few minutes to pipette HD or CEES into the medium, and then into a multi-well plate. During this time hydrolysis will not only lower the "real" concentration of CEES but also make the effective CEES concentration different from the first to the last sample. Therefore, a more stable stock solution of CEES in a dry organic solvent would be preferable. Previously, we showed that DMSO was a very effective vehicle for CEES delivery to the cells. We found, also, that DMSO is more effective vehicle than ethanol. CEES/DMSO stock solution provides a better application model than direct dispersing of CEES in a large bolus of cell culture medium prior the experiment.

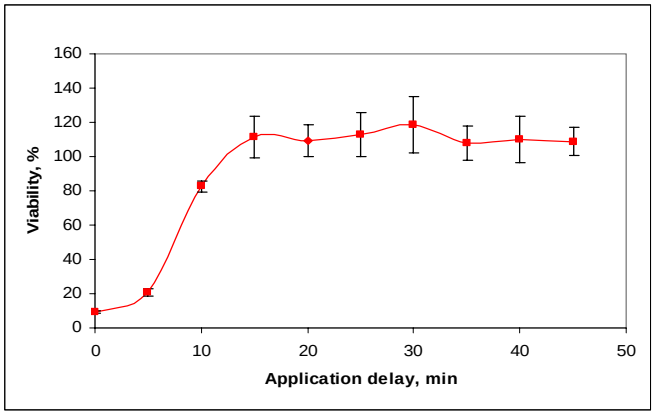

Figure 1. Influence of CEES Hydrolysis on Its Toxicity. 1.5 mM CEES in media was applied to HaCaT cells in 96-well plate after various time delays, as indicated. Cell viability was measured by the MTT assay after 24 hours.

In order to determine if CEES hydrolysis in culture medium has an effect on its toxicity, we performed a simple time-course experiment. CEES (50 mM stock in DMSO) was mixed with

appropriate volume of media to obtain a 1.5 mM final concentration, and then applied to HaCaT keratinocytes in a 96-well plate after various time delays. As shown in **Figure 1**, a quick drop in CEES toxicity was noted with only a 5 min delay. After 15 minutes, CEES was no longer toxic to the cells. The data support view that CEES hydrolysis is a critical experimental parameter in cellular toxicity studies.

Although we found that DMSO itself possesses a mild cytotoxic effect, such drawback can be minimized by using a low vehicle concentration (1% (vol./vol.)). We have preformed a number of experiments to optimize the CEES application technique. Our results indicate that preparing a 100x stock solution in DMSO followed by quick mixing with media for only a limited number of repeated samples (up to 8) is very effective. We found that CEES hydrolysis is minimal with this technique (data not shown).

Figures 2 shows comparison of various applicati

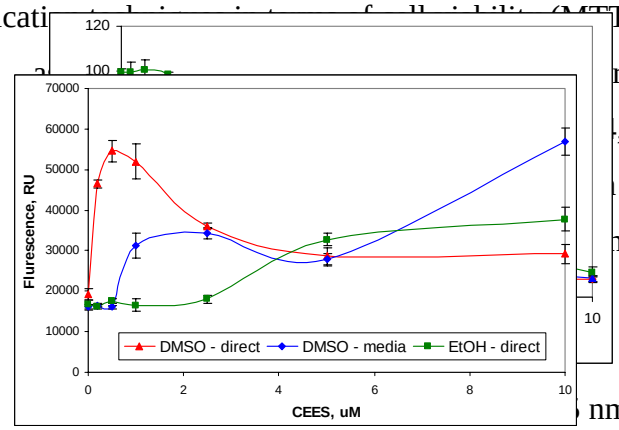

Figure 2. Influence of CEES Application Technique on Cell Viability. CEES (0.2 - 10 mM) was applied to HaCaT cells in a 96-well plate: (1) directly as a stock solution in DMSO, (2) directly as a stock solution in EtOH, (3) as a mixture of DMSO and media. **Left:** Cell viability was measured by the MTT assay after 24 hours. **Right:** Amount of dead cells was measured by the PI assay after 24 hours.

(propidium iodide (PI) dye to differentiate live and dead cells. Cells that have lost membrane integrity cannot exclude PI, which emits a red fluoresce after binding to nuclear DNA or double stranded RNA. PI fluorescence was measured using a Fluostar Galaxy microplate reader using an excitation wavelength of 485 nm and an emission wavelength of 650 nm.

Applying CEES in DMSO directly to the well as cultured cells resulted in maximum loss of cell viability (**Figure 2, left**) and maximum cell death (**Figure 2, right**). This is due to minimizing CEES hydrolysis as exposing the cells to a transiently high level of CEES. The transiently high level of CEES is an uncontrolled variable and this fact minimizes the usefulness of direct application of stock CEES in DMSO. Adding the CEES in DMSO followed by rapid mixing in the media also produces marked loss of viability and increased cell death. This method has the advantage of not exposing the cells to a transiently high level of CEES and provides reproducible results with an IC_{50} of about 1 mM, which is in agreement with other studies [18-20]. Applying CEES directly in ethanol was effective at reducing cell viability and inducing cell death.

In order to determine if an addition of immuno-stimulators (LPS, PMA) and pro-inflammatory cytokines (TNF-α, IL-1β) increase CEES toxicity in immortalized HaCaT keratinocytes, we performed additional experiments with these cells exposed to various levels of CEES. None of these immuno-stimulators enhanced toxicity of CEES in NHEK keratinocytes as we reported previously. However, in proliferating HaCaT cells TNF-α did show dose-dependent enhancement of CEES toxicity (**Figure 3)**, although LPS and IL-1β did not have any significant effect (data not shown)**.** The difference between NHEK and HaCaT cell reaction to the TNF-α treatment can be explained by the fact that HaCaT keratinocytes, unlike NHEK cells, proliferate continuously and do not undergo terminal differentiation. As a consequence, HaCaT cells much more susceptible to the CEES/HD toxicity. The latter results are in agreement with our previous

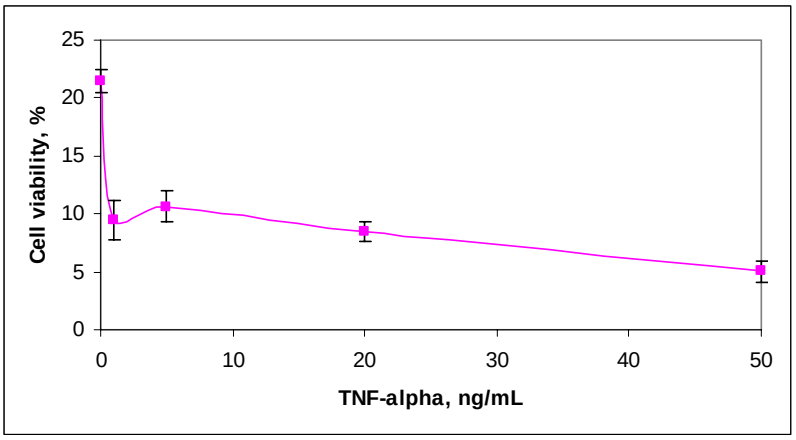

Figure 3. TNF Attenuates CEES Toxicity in Human Keratinocytes. HaCaT cells were treated with 1 mM CEES and various levels of TNF-alpha (simultaneously). Cell viability was measured by the CAM assay after 24 hours.

observations obtained with murine macrophages simultaneously exposed to CEES and various immuno-stimulators [5].

Using the direct CEES/DMSO application, we studied the cell death mechanisms in CCD 1106 KERTr keratinocytes. As a first step, we evaluated apoptosis as measured by the activation of caspase 3 activity in response to various levels of CEES after 24 hours (**Figure 4**). In this experiment CEES toxicity was relatively high, as we did not pre-mix the stock solution with media, but added it directly into a well which permits a transiently high local concentration of CEES/DMSO to exist before mixing is complete. We found that the low doses of CEES (50 - 100 µM) induced apoptosis then higher doses did not. These data suggest that necrosis, rather than apoptosis, was the cause of cell death at the higher concentration. Staurosporin, a broad-spectrum kinase inhibitor, was used as a positive control since it is a well known caspase activator and apoptosis inducer.

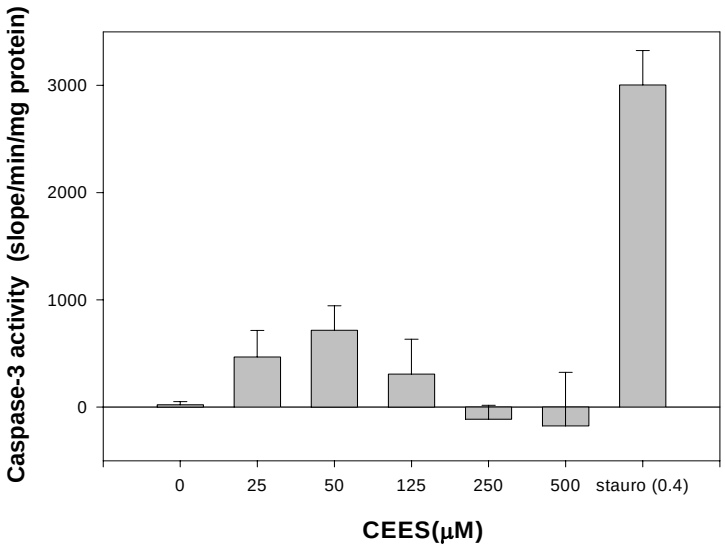

Figure 4. CEES-mediated Apoptosis in Human Keratinocytes. CEES (0.025 – 0.5 mM) was applied to CCD 1106 cells in a 96-well as a stock solution in DMSO. Apoptosis was measured after 24 hours as caspase-3 activity in cell lysates normalized to protein levels. Staurosporin (0.4 uM) was used as a positive control.

We next examined the time course for apoptosis at the low doses of CEES. As shown in **Figure 5,** we found that CEES induced activation of caspase 3 was maximal after 12 hours of incubation, which is in agreement with other reports regarding CEES or HD toxicity in human skin cells [18, 21, 22].

Specific Task 2:

As CEES induces caspase-dependant apoptotic cell death in human keratinocytes it would be important to explore the relationship between apoptosis and oxidative stress in these cells. In order to explore oxidative stress in CEES treated keratinocytes, we stained HaCaT cells incubated with 2 mM CEES for 8 hours with carboxy-dichloroflurescin diaceatate (Car-DCFH DA), a

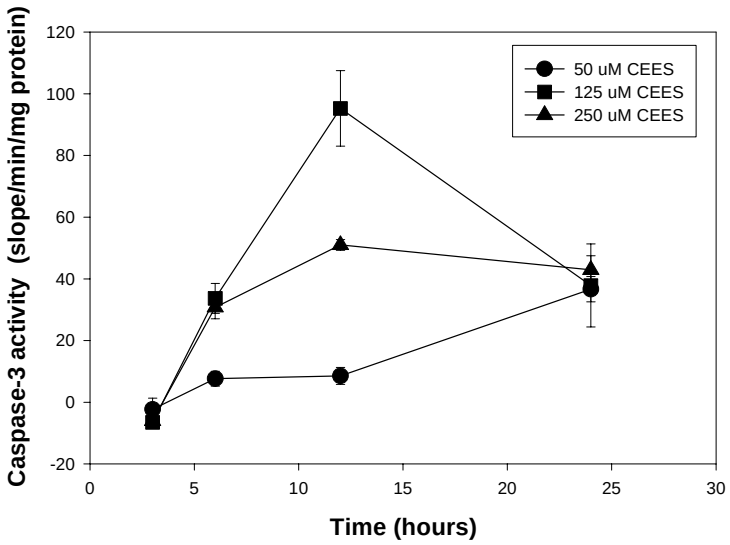

Figure 5. CEES-mediated Apoptosis in Human Keratinocytes. CEES (0.05 – 0.25 mM) was applied to CCD 1106 cells in a 96-well as a stock solution in DMSO. Apoptosis was measured after 24 hours as caspase-3 activity in cell lysates normalized to protein levels.

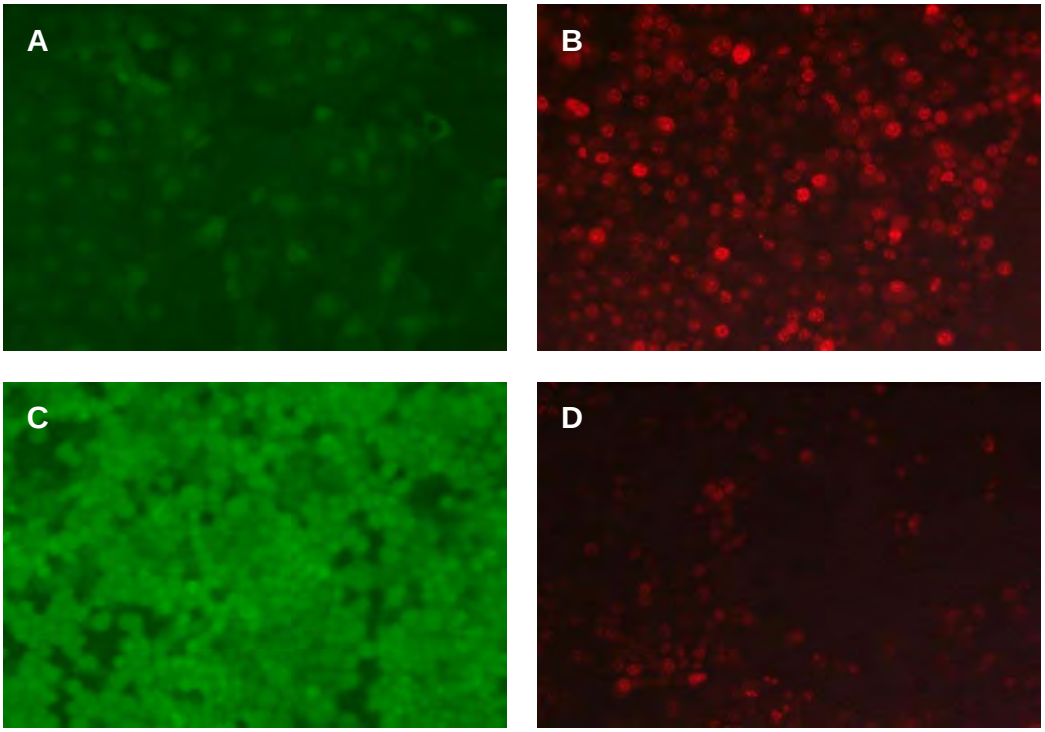

Figure 6. Oxidative Stress in Human Keratinocytes. HaCaT cells were incubated with 2 mM CEES (A, B) or 0.1 mM TBHP (C,D) for 8 hours in a 96-well plate. Oxidative stress was monitored by Car-DCFH DA staining (green fluorescence, on the left); cell death was monitored by PI staining (red fluorescence, on the right) under fluorescent microscope.

membrane-permeable dye sensitive to reactive oxygen species (ROS) in the cytosol. The cells were simultaneously stained with propidium iodide (PI), which is membrane-impermeable DNA sensitive dye, and marks nuclei of dead cells. **Figure 6B** shows cell death after CEES treatment as most of the nuclei are stained with PI; however, Car-DCFH DA staining did not reveal massive ROS production in these cells (**Figure 6A**). Figure 1C and D show a positive control, HaCaT cells incubated with 0.1 mM tert-butyl hydroperoxide (TBHP). In this case, Car-DCFH DA staining resulted in 4 fold higher fluorescence indicating massive generation of hydrogen peroxide and other ROS; however, cell survival was much greater in comparison with CEES treatment (**Figure 6C and D**, respectively). Although we did not detect oxidative stress changes in CEES treated keratinocytes in this experiment, we believe that the present data do not provide sufficient evidence to make a final conclusion. We will repeat similar experiments using other markers of oxidative stress, such as dihydroxyethidium and dihydrorhodamine 123.

Although the direct measurement of ROS generation during CEES treatment was not yet successful in our experiments, we found indirect evidence for oxidative stress related changes in the experiments presented in the **Figure 7**. It is known that HD/CEES toxicity is associated with glutathione depletion, which is the major cytosolic antioxidant. Such depletion greatly reduces anti-oxidative potential of the cell, making it susceptible even to moderate oxidative changes. In order to explore the sensitivity of CEES treated cells to exogenous oxidative stimuli we examined CEES mediated toxicity in the presence or absence of TBHP, an agent promoting massive intracellular ROS generation.

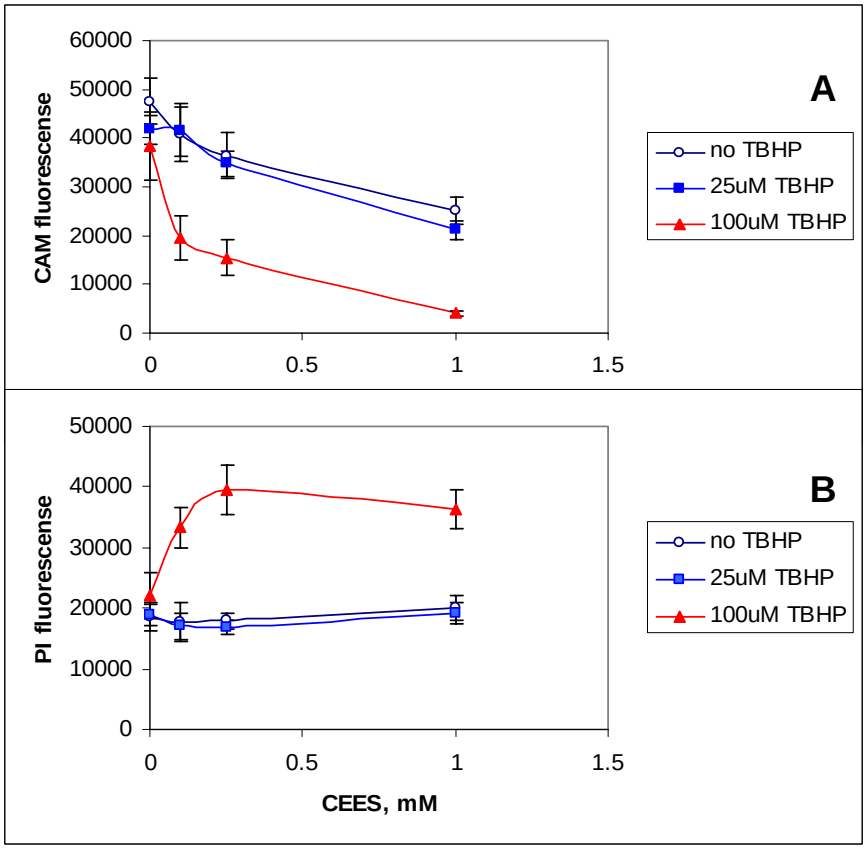

Figure 7. Oxidative Stress Enhances CEES Toxicity in Human Keratinocytes. HaCaT cells were incubated with CEES (as indicated) in the presence or absence of 25μM or 100 μM TBHP for 24 hours in a 96-well plate. Cell viability was monitored by calcein AM (CAM) staining (panel A), cell death was monitored by PI staining (panel B).

Interestingly, non-toxic doses of TBHP (up to 0.1 mM) significantly increased the toxic effect of CEES as measured via calcein AM (CAM) and PI staining (**Figure 7A and 7B**, respectively). CAM is a fluorescent marker of viable cells, whereas PI is a marker of dead cells. The data obtained with CAM staining were also confirmed with MTT cell viability assay (data not

shown). This effect of CEES is similar to our earlier observation in murine macrophages, where lipopolysaccaride (LPS) enhanced toxicity of the CEES. LPS is a potent inducer of oxidative stress in immune cells [5]. We believe that CEES mediated sensitivity to oxidative stress is important in HD/CEES toxicity *in vivo*.

Whether CEES directly induces ROS generation in keratinocytes is still unclear. We cannot find any evidence of such effect in the literature. However, HD is well known to induce ROS generation in endothelial and immune cells [23, 24]. Thus, oxidative stress may occur *in vivo* as a result of immune cell attack in response to keratinocyte-derived pro-inflammatory cytokines, such as TNF-α [9]. To explore this possibility in the future, we plan a multi-cell type experiment with CEES treated keratinocytes and non-treated macrophages.

Specific Task 3:

Another indirect evidence for the role of oxidative stress in HD/CEES toxicity is the protective effect of NAC. This potent antioxidant is capable of restoring intracellular GSH level, and has already been shown to be protective against CEES toxicity *in vivo* [25]. We have extensively studied the effect of NAC in CEES treated human keratinocytes. Using two independent caspase-3 assays we have shown that NAC effectively down-regulates CEES induced apoptosis in HaCaT cells. NAC (10 mM) reduces caspase-3 activity after 12 hour incubation (**Figure 8**). Caspase-3 activity was measured in cell lysates using the fluorescent substrate peptido (DEVD)-7-Amino-4-trifluoromethylcoumarin (DEVD-AFC) and normalized to the total cellular protein. We also performed Live Cell NucView 488 Caspase-3 assay, which allows detection of active caspase-3 in living cells. NucView 488 Caspase-3 substrate permeates the cell and undergoes specific enzymatic cleavage by caspase-3; the product is fluorescent and binds nuclear DNA. Thus, this method not only marks viable cells with activated caspase-3, but also reveals chromatin condensation in the apoptotic nuclei.

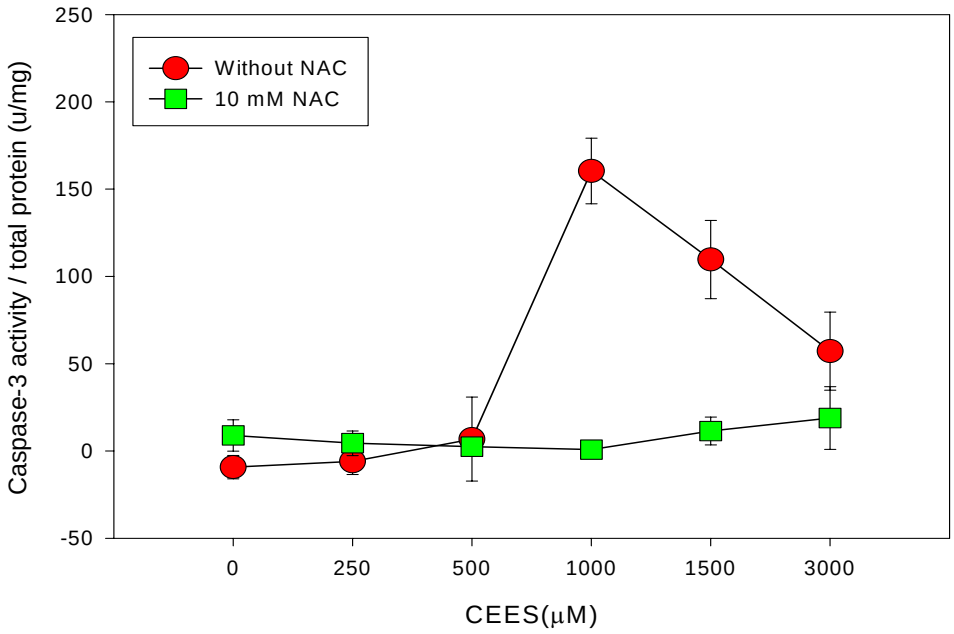

Figure 8. NAC Effect on CEES Induced Apoptosis in Human Keratinocytes. HaCaT cells were incubated with CEES (as indicated) in the presence or absence of 10 mM NAC for 24 h. Caspase-3 activity was measured in the cell lysates using DEVD-AFC substrate and normalized to the total protein.

Figure 9 shows CEES induced apoptosis in human keratinocytes. CEES (2 mM) promoted intensive green staining in HaCaT cells after 18 hours (**Figure 9C**); whereas similar incubation with 1% DMSO (vehicle) had no effect (**Figure 9A**). The green background reflects a phase-contrast view of the cells, which was merged with the fluorescent image. **Figure 9B and 9D** show positive (5 µM staurosporine) and negative controls (5 µM staurosporine with caspase-3 inhibitor), respectively.

Figure 10 shows the protective effect of NAC against CEES induced apoptosis in human keratinocytes. **Figure 10A and 10B** display CEES induced caspase-3 activation after 18 hours. Green fluorescence marks caspase-cleaved fluorescent product as it stains apoptotic nuclei; blue fluorescence (DAPI) stains nuclei of apoptotic cells. Markers at the pictures point to nuclei with clear visible chromatin condensation. Although NAC does not completely prevent CEES induced apoptosis (**Figure 10C and 10D**), it reduces the number of apoptotic cells as it can be

seen with intense DAPI staining. Phase-contrast

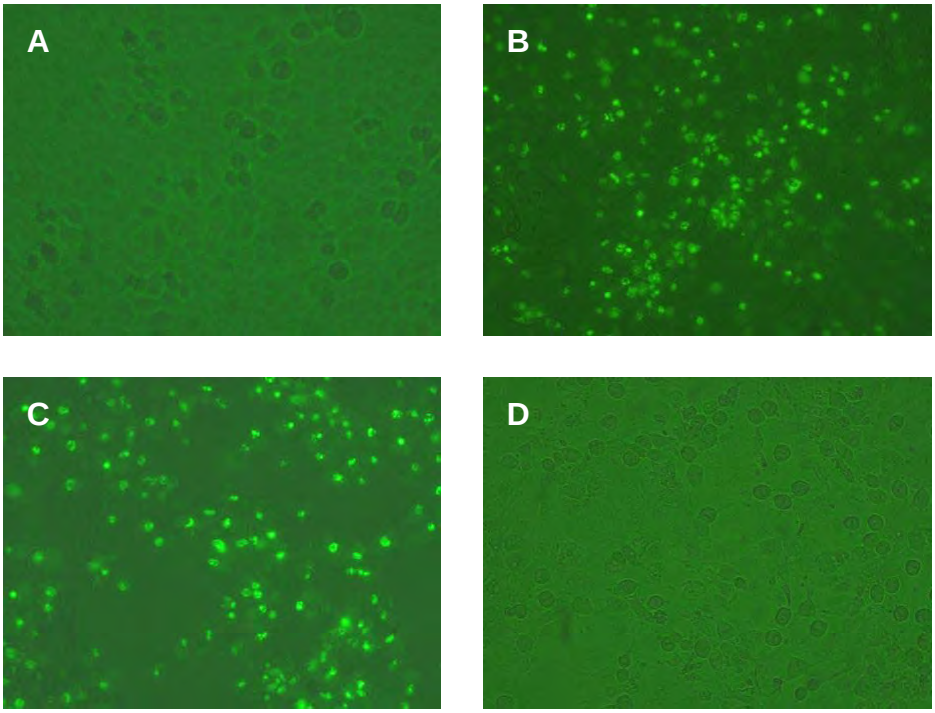

Figure 9. CEES-induced Apoptosis in Human Keratinocytes. HaCaT cells were incubated with vehicle (A), or 5μM staursporine (B), or 2 mM CEES (C), or 5μM staursporine with caspase-3 inhibitor (D) for 18 h. Caspase-3 activity was monitored using Live Cell NucView 488 Caspase-3 assay kit under fluorescent microscope.

images of the same cells revealed dense confluent cell layers for both CEES and CEES/NAC treatments (not shown).

We also measured the protective effect of NAC quantitatively using MTT assay. **Figure 11A** shows that 5 mM NAC significantly increased cell viability if applied simultaneously with 2mM CEES. **Figure 11B** shows the increased cell viability of CEES treated keratinocytes as a function of NAC concentration. Notably, neither pre-treatment nor post-treatment of the CEES treated HaCaT cells with 10 mM NAC (cells were incubated 5 hours prior or after the CEES application) had any protective effect (data not shown).

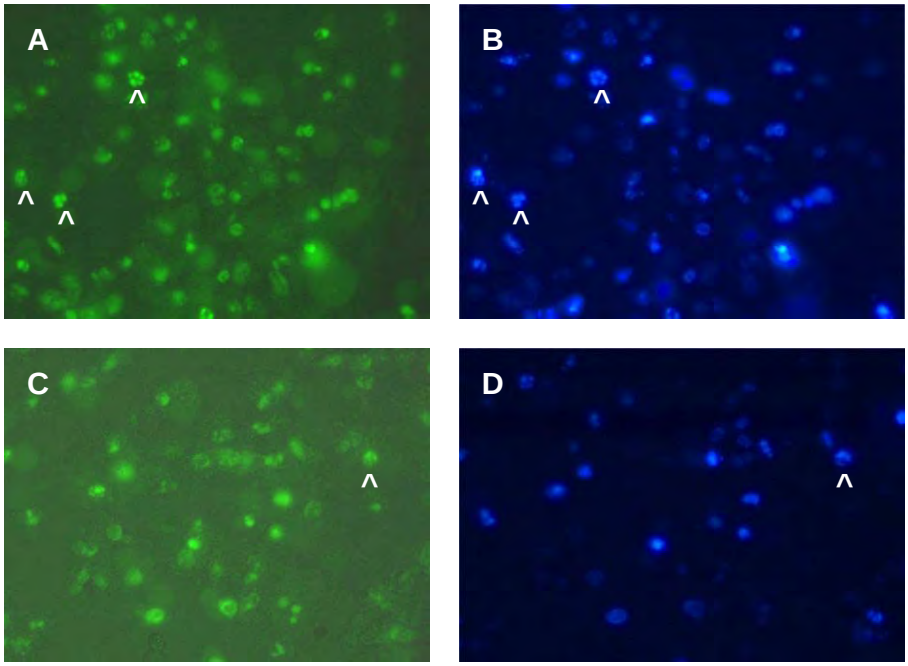

Figure 10. NAC Protects against CEES-induced Apoptosis in Human Keratinocytes. HaCaT cells were incubated with 2 mM CEES in the absence (A, B) or presence (C, D) of 10 mM NAC for 18 h. Caspase-3 activity was monitored using Live Cell NucView 488 Caspase-3 assay kit (green, left panels) under fluorescent microscope. Excessive DAPI staining (blue, right panels) shows apoptotic nuclei. Markers point to apoptotic nuclei with condensed chromatin.

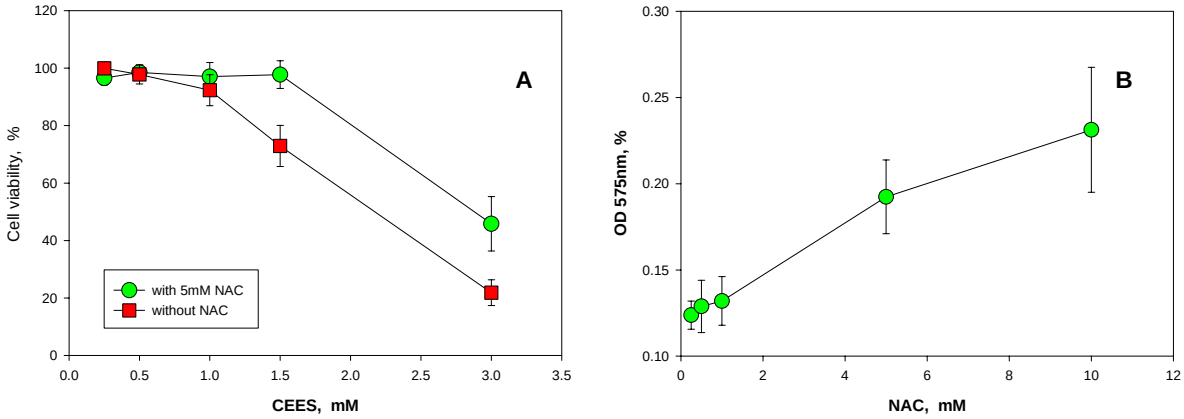

Figure 11. NAC Protects against CEES-induced Toxicity in Human Keratinocytes. HaCaT cells were incubated with CEES (as indicated) in the absence or presence of 5 mM NAC (panel A). HaCaT cells were incubated with 2 mM CEES in the presence of NAC (as indicated) (panel B). NAC was added to the media immediately after the CEES application. Cell viability was measured via standard MTT assay after 24 hours.

Specific Task 4:

Using CEES/DMSO application, we have tested five different types of antioxidant liposomes (see **Table 1**) for their ability to inhibit CEES toxicity in HaCaT keratinocytes. The liposomal formulations were: (1) **Blank liposomes** (no water- or lipid-soluble antioxidants); (2) glutathione containing **GSH-liposomes** (75 mM GSH); (3) alpha-tocopherol containing **T-liposomes** (3.33 mM alpha-tocopherol); (4) alpha-tocopherol and GSH containing **G/T-liposomes** (3.33 mM alpha-tocopherol with 75 mM GSH); (5) alpha/gamma-tocopherol and NAC containing **NAC/T-liposomes** (3.1 mM alpha-tocopherol, 3.1 mM gamma-tocopherol with 75 mM NAC). **Figure 12** shows that the NAC/T liposomes partially prevented 1mM CEES-induced loss of cell viability, as measured by an MTT assay, whereas none of the other liposomal formulations were effective.

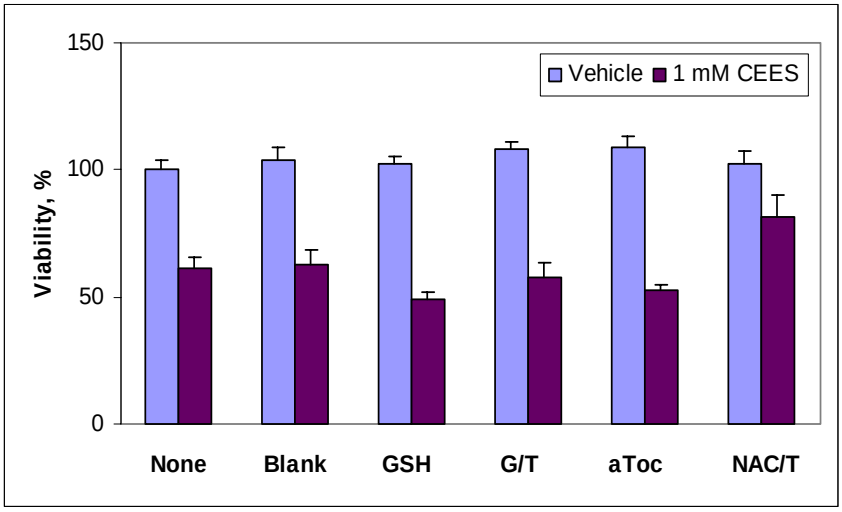

Figure 12. Antioxidant Liposome Protection against CEES Toxicity. CEES (1 mM) was applied as a stock solution in DMSO pre-mixed with media. Various liposomes were added immediately after the CEES application. Cell viability was measured by the MTT assay after 24 hours. **None:** cells treated with DMSO or CEES in the absence of liposomes; **Blank:** Blank liposomes; **GSH:** GSH-liposomes; **G/T:** GSH/alpha-tocopherol-liposomes; **aToc:** alpha-tocopherol-liposomes; **NAC/T:** NAC/alpha-tocopherol-liposomes.

We later examined the protective abilities of NAC-containing liposomes against CEES toxicity in a series of separate experiments. We treated HaCaT cells with CEES and antioxidant liposomes simultaneously. **Figure 13** shows a protective effect of various concentrations of **NAC-liposomes** in comparison with blank liposomes in HaCaT cells. Both cell proliferation ability (CAM assay) and cell death (PI assay) were monitored after 24 hour of incubation with 2 mM CEES/DMSO. **NAC-liposomes (NL)** showed

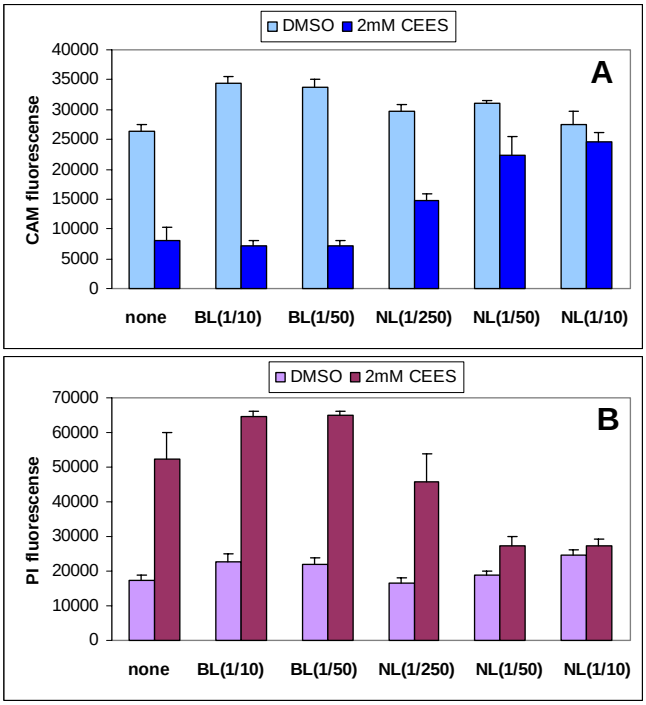

Figure 13. NAC Liposomes Protect against CEES-induced Toxicity in Human Keratinocytes. HaCaT cells were treated with 2 mM CEES; NAC liposomes (NL) or Blank liposomes (BL) were added simultaneously with CEES. Cell viability was monitored by CAM staining (panel A), cell death was monitored by PI staining (panel B) after 24 hours. None: cells treated with DMSO or CEES in the absence of liposomes; numbers show final dilutions of the liposomes.

protective effect in HaCaT keratinocytes treated with CEES, which was dose-dependent. **Blank liposomes (BL)** made with lipids only (negative control) showed no protection, as expected. Liposome samples were diluted in media as indicated. Dilutions of 1:10, 1:50, and 1:250 gave 7.5 mM, 1.5 mM, and 0.3 mM final concentrations of NAC, respectively.

These data are particularly encouraging since they fully support our hypothesis that CEES (and HD) pathophysiology is mediated, at least in part, by oxidative stress and that antioxidant-liposomes represent an effective counter measure.

In addition, we found recently that mitochondria substrates, such as such as pyruvates, can be protective against CEES/HD mediated oxidative stress and cell death. A number of studies have shown that mitochondria substrates, attenuate mitochondria dysfunction and prevent cell death in

various types of human cells exposed to alkylating agents, such

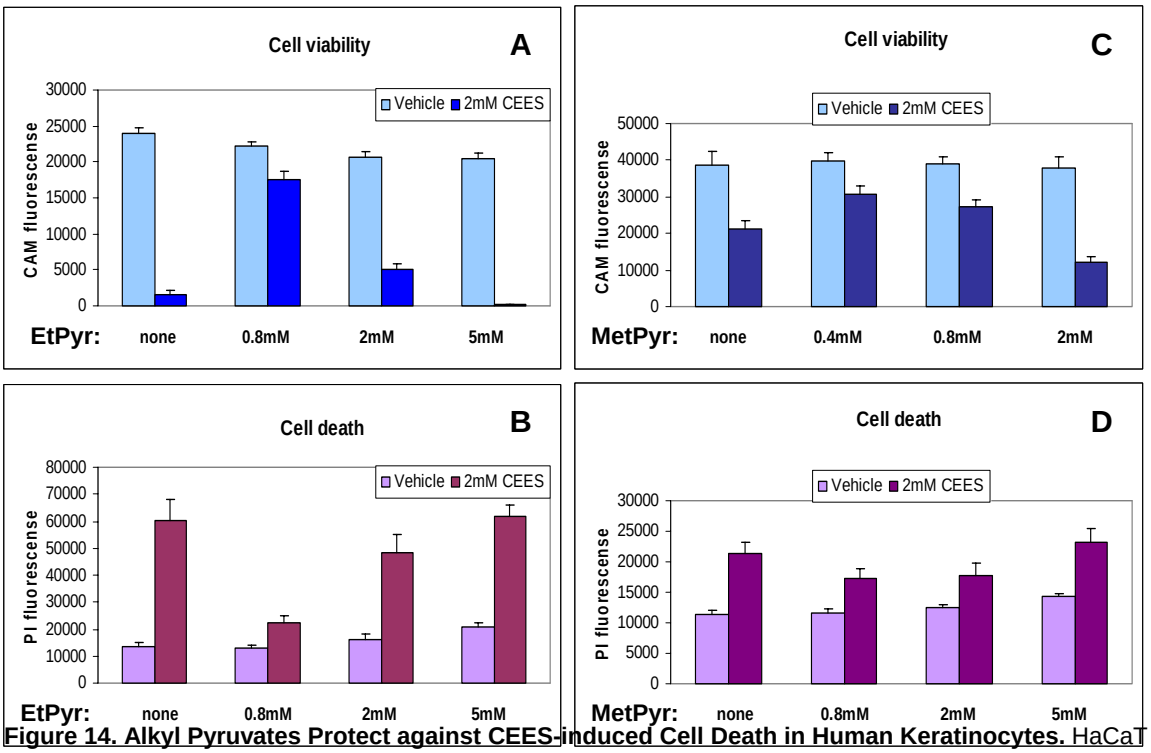

Figure 14. Alkyl Pyruvates Protect against CEES-induced Cell Death in Human Keratinocytes. HaCaT cells were incubated with 2 mM CEES or vehicle (DMSO) in the absence (none) or presence of methyl pyruvate (MetPyr; Panels A, B) or ethyl pyruvate (EtPyr; Panels C, D) (as indicated) for 24 h. Cell viability was monitored via CAM fluorescent staining; cell death was monitored via PI fluorescent staining.

nitrogen mustard and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), chemical analogs of HD used as anti-cancer drugs[26-28]. Therefore, we initiated a series of experiments aimed to explore the possible protective effect of pyruvates (methyl pyruvate and ethyl pyruvate) on HaCaT keratinocytes exposed to HD. In case pyruvates would prove their protective abilities against CEES in human keratinocytes antioxidant liposomes containing them will be formulated in tested in the EpiDerm™ human skin model.

Figure 14 shows the protective effect of methyl pyruvate (Panel A) or ethyl pyruvate (Panel B) in HaCaT cells exposed to 2 mM CEES or vehicle (DMSO). Hank's Balanced Salt Solution (Sigma) supplied with 5% FBS was used instead of media as Keratinocyte Media from Sigma, GibCo, or Cambrex contain 0.5 mM sodium pyruvate. The results revealed that simultaneous application of CEES and a low level of pyruvate (0.2 – 2 mM) would sufficiently reduce the toxicity of CEES both in assays measuring cell viability (CAM assay) and the ones monitoring cell death (PI assay). These results will be further re-evaluated in order to find an optimal pyruvate concentration and possibly formulate antioxidant liposomes containing pyruvate for studies in the EpiDerm™ human skin model.

Table 1. Chemical and Physical Characterization of Liposomes

Type of Liposomes					
Blank	**GSH**	**AT**		**GSH/AT**	**NAC/T**
Lipid Components	**Mole Fractions/Concentration**				
PL/type	0.71/85G	0.71/85G	0.67/85G	0.67/85G	0.62/90H
PA	0	0	0	0	0.006
cholesterol	0.29	0.29	0.27	0.27	0.248
AT/mM	0/0	0/0	0.07/3.33	0.07/3.33	0.062/3.1
GT/mM	0/0	0/0	0/0	0/0	0.062/3.1
Aqueous Components					
GSH/mM	0	75	0	75	0
NAC/mM	0	0	0	0	75
Size (nm)/% Peak 1	29.6±5.3 8%	90.2±16 23.5%	74.2±15.4 34.6%	78.6±13.4 37.2%	152.1±31.4 37.2%
Size (nm)/% Peak 2	141.8±28.5 92%	190.3±41.2 69.3%	248.5±45.8 65.4%	273.9±61.2 62.8	1326.3±292.6 68.6%

Specific Task 5:

The experiments for optimization of the antioxidant liposome large-scale production and long-term storage conditions have been reported in the previous yearly Report.

KEY RESEARCH ACCOMPLISHMENTS:

- □ Various aspects of CEES toxicity in human keratinocytes (inhibition of cell growth, oxidative stress parameters, influence of various immuno-stimulators) have been studied
- □ CEES application techniques to the cells have been optimized in order to provide a means of dealing with the rapid hydrolysis of CEES as an experimental variable
- □ Enhancement of CEES induced toxicity in HaCaT cells in the presence of TNF-α was documented
- □ We have confirmed that CEES induces apoptosis in human keratinocytes as measured by the caspase 3 assay; apoptotic changes were shown in living cells
- □ Oxidative stress related changes were studied in HaCaT cells using a fluorescent staining technique
- □ Enhancement of CEES induced toxicity in HaCaT cells was observed using an oxidative stress inducer TBHP
- □ Protective effect of NAC was studied in HaCaT cells. Effective concentrations of NAC have been shown to reduce CEES induced apoptosis and increase cell survival
- □ Major types of antioxidant liposomes have been tested in prevention of CEES-induced cell damage in human keratinocytes; protective effects have been documented
- □ The alpha-tocopherol and NAC-containing liposomes have been found to be most effective in preventing CEES induced loss of keratinocyte viability and CEES induced cell death of keratinocytes
- □ NAC containing liposomes were shown to protect HaCaT cells against CEES induced toxicity in dose-dependent manner

- Ethyl pyruvate and methyl pyruvate have been shown to HaCaT keratinocytes against CEES induced toxicity in dose-dependent manner

REPORTABLE OUTCOMES:

Publications:

Viktor Paromov, Milton Smith, and William L. Stone "Sulfur Mustard Toxicity in Human Skin: Role of Oxidative Stress, and Antioxidant Therapy" in: *Journal of Burns and Wounds* (submitted).

Viktor Paromov, Min Qui, Hongsong Yang, and Milton Smith, William L. Stone "The influence of N-acetyl-L-cysteine and Polymyxin B on oxidative stress and nitric oxide synthesis in stimulated macrophages treated with a mustard gas analog" in: *BMC Cell Biology* (submitted).

Min Qui, Viktor Paromov, Hongsong Yang, Milton Smith, and William L. Stone "Inhibition of inducible Nitric Oxide Synthase by a mustard gas analog in murine macrophages", in: *BMC Cell Biology* **7**, 39

Suntres, Z, Stone, WL, and Smith, MG: Ricin-Induced Toxicity: The Role of Oxidative Stress, in: *J Med CBR Defense*, 3 (2005).

Smith, M, Das, S, Ward, P, Suntres, Z, Crawford, K and Stone, WL: Blister Agents and Oxidative Stress, In: *CHEMICAL WARFARE AGENTS: Chemistry, Pharmacology, Toxicology and Therapeutics*, CRC Press (in preparation)

Presentations/Abstracts:

Hoesel, L.M., Pianko, M.J., Yang, H., Stone, W.L., Smith, M.G., Ward, P.A., Liposomes Containing Antioxidants Prevent Pulmonary Fibrosis in Half-Sulfur Mustard Gas Induced Lung Injury, Bioscience Review 2006, sponsored by the US Army Medical Research and Material Command and hosted by the US Army Medical Research Institute of Chemical Defense, June 4-9, 2006, Hunt Valley, invited platform presentation and abstract (page 109).

Stone, W.L., Li, Q., Paromov, V., Qui, M., Yang, H., Smith, M., Antioxidant Liposome Therapy for Exposure to a Sulfur Vesicating Agent, Bioscience Review 2006, sponsored by the US Army Medical Research and Material Command and hosted by the US Army Medical Research

Institute of Chemical Defense, June 4-9, 2006, Hunt Valley, poster presentation and abstract (page 161).

Smith, M.G., Stone, W.L., Ward, P., Alibek, K., Wu, A., Das, S., Crawford, K., Anderson, D., Sciuto, A., Suntres, Z., Rest, R., A Multi-Threat and Diagnostic Countermeasure by the Advanced Medical Countermeasure Consortium, Bioscience Review 2006, sponsored by the US Army Medical Research and Material Command and hosted by the US Army Medical Research Institute of Chemical Defense, June 4-9, 2006, Hunt Valley, poster presentation and abstract (page 259).

Degrees obtained that are supported by this award: Mr. Christian Muenyi, graduate student in PI's lab has received MS degree from the Chemistry Department at East Tennessee State University as he initiated proteomic studies in order to complement the work funded by this application.

Funding applied for based on work supported by this award: NA

Employment or research opportunities applied for and/or received based on experience/training supported by this award: NA

Invited Presentations at International Meetings: The experiments described in this report have been presented as a poster at the Bioscience Review 2006 International Conference, Hunt Valley, Maryland (see attached abstract). In addition, Dr. Hongsong Yang has been invited to present our finding at the 6th International Workshop of Micronutrients, Oxidative Stress and the Environment held in Kuching, Malaysia, June 29th –July 2nd, 2006.

CONCLUSION:

Our experiments further demonstrated CEES-induced oxidative stress and caspase-dependent apoptosis in human keratinocytes. Apoptotic changes were shown via fluorescent imaging in living cells. Although we did not find an evidence of a direct CEES-induced generation of ROS in human keratinocytes (as it was documented in other cell types), in these cells, we did register a dose-dependent enhancement of CEES toxicity by the TBHP-induced oxidative stress. We also found that pro-inflammatory cytokine TNF-α is able to enhance CEES-induced cytotoxicity in a dose-dependent manner. The latter results are in agreement with our previous observations obtained with murine macrophages simultaneously exposed to CEES and various immuno-stimulators [5].

We further studied protective effect of NAC in human keratinocytes. Effective concentrations of NAC have been shown to reduce CEES-induced apoptosis and increase cell survival in a dose-dependent manner.

We continued to study the protective effect of antioxidant liposomes against CEES-induced skin cell damage. In particular, NAC/α-tocopherol containing liposomes showed significant protective effect, and NAC-containing liposomes have been found to be most effective in preventing CEES-induced loss of keratinocyte viability. Also, NAC-containing liposomes were shown to protect HaCaT cells against CEES-induced toxicity in a dose-dependent manner.

In addition, we found that mitochondria substrates, such as such as pyruvates, can be protective against CEES-mediated oxidative stress and cell death. Ethyl pyruvate and methyl pyruvate have been shown to protect HaCaT keratinocytes against CEES-induced toxicity in a dose-dependent manner.

REFERENCES:

1. Davreux CJ, Soric I, Nathens AB, Watson RW, McGilvray ID, Suntres ZE, Shek PN, Rotstein OD: **N-acetyl cysteine attenuates acute lung injury in the rat.** *Shock* 1997, **8:**432-438.
2. Fox ES, Brower JS, Bellezzo JM, Leingang KA: **N-acetylcysteine and alpha-tocopherol reverse the inflammatory response in activated rat Kupffer cells.** *J Immunol* 1997, **158:**5418-5423.
3. Fu Y, McCormick CC, Roneker C, Lei XG: **Lipopolysaccharide and interferon-gamma-induced nitric oxide production and protein oxidation in mouse peritoneal macrophages are affected by glutathione peroxidase-1 gene knockout.** *Free Radical Biology & Medicine* 2001, **31:**450-459.
4. Wang S, Leonard SS, Castranova V, Vallyathan V, Shi X: **The role of superoxide radical in TNF-alpha induced NF-kappaB activation.** *Ann Clin Lab Sci* 1999, **29:**192-199.
5. Stone WL, Qui M, Smith M: **Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide.** *BMC Cell Biol* 2003, **4:**1.
6. Mercurio F, Manning AM: **NF-kappaB as a primary regulator of the stress response.** *Oncogene* 1999, **18:**6163-6171.
7. Mercurio F, Manning AM: **Multiple signals converging on NF-kappaB.** *Curr Opin Cell Biol* 1999, **11:**226-232.
8. Downey JS, Han J: **Cellular activation mechanisms in septic shock.** *Front Biosci* 1998, **3:**d468-476.
9. Arroyo CM, Schafer RJ, Kurt EM, Broomfield CA, Carmichael AJ: **Response of normal human keratinocytes to sulfur mustard: cytokine release.** *Journal of Applied Toxicology : Jat* 2000, **20 Suppl 1:**S63-72.

10. Chatterjee D, Mukherjee S, Smith MG, Das SK: **Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustard analog.** *J Biochem Mol Toxicol* 2003, **17:**114-121.
11. Atkins KB, Lodhi IJ, Hurley LL, Hinshaw DB: **N-acetylcysteine and endothelial cell injury by sulfur mustard.** *J Appl Toxicol* 2000, **20 Suppl 1:**S125-128.
12. Pathania V, Syal N, Pathak CM, Khanduja KL: **Vitamin E suppresses the induction of reactive oxygen species release by lipopolysaccharide, interleukin-1beta and tumor necrosis factor- alpha in rat alveolar macrophages.** *J Nutr Sci Vitaminol (Tokyo)* 1999, **45:**675-686.
13. Thirunavukkarasu V, Anuradha CV: **Influence of alpha-lipoic acid on lipid peroxidation and antioxidant defence system in blood of insulin-resistant rats.** *Diabetes Obes Metab* 2004, **6:**200-207.
14. Tsuchiya M, Kagan VE, Freisleben HJ, Manabe M, Packer L: **Antioxidant activity of alpha-tocopherol, beta-carotene, and ubiquinol in membranes: cis-parinaric acid-incorporated liposomes.** *Methods Enzymol* 1994, **234:**371-383.
15. Stone WL, Mukherjee S, Smith M, Das SK: **Therapeutic uses of antioxidant liposomes.** *Methods Mol Biol* 2002, **199:**145-161.
16. Kim YB, Lee YS, Choi DS, Cha SH, Sok DE: **Change in glutathione S-transferase and glyceraldehyde-3-phosphate dehydrogenase activities in the organs of mice treated with 2-chloroethyl ethyl sulfide or its oxidation products.** *Food Chem Toxicol* 1996, **34:**259-265.
17. Rohrbaugh DK, Yang YC, Ward JR: **Identification of degradation products of 2-chloroethyl ethyl sulfide by gas chromatography-mass spectrometry.** *J Chromatogr* 1988, **447:**165-169.
18. Blaha M, Kohl J, DuBose D, Bowers W, Jr., Walker J: **Ultrastructural and histological effects of exposure to CEES or heat in a human epidermal model.** *In Vitr Mol Toxicol* 2001, **14:**15-23.
19. Ray P, Ali ST: **Protease in normal human epidermal keratinocytes.** *Drug Chem Toxicol* 1998, **21:**319-327.
20. Chatterjee D, Mukherjee S, Smith MG, Das SK: **Evidence of hair loss after subacute exposure to 2-chloroethyl ethyl sulfide, a mustard analog, and beneficial effects of N-acetyl cysteine.** *J Biochem Mol Toxicol* 2004, **18:**150-153.
21. Ray R, Hauck S, Kramer R, Benton B: **A convenient fluorometric method to study sulfur mustard-induced apoptosis in human epidermal keratinocytes monolayer microplate culture.** *Drug Chem Toxicol* 2005, **28:**105-116.
22. Rosenthal DS, Velena A, Chou FP, Schlegel R, Ray R, Benton B, Anderson D, Smith WJ, Simbulan-Rosenthal CM: **Expression of dominant-negative Fas-associated death domain blocks human keratinocyte apoptosis and vesication induced by sulfur mustard.** *J Biol Chem* 2003, **278:**8531-8540.
23. Levitt JM, Lodhi IJ, Nguyen PK, Ngo V, Clift R, Hinshaw DB, Sweeney JF: **Low-dose sulfur mustard primes oxidative function and induces apoptosis in human polymorphonuclear leukocytes.** *Int Immunopharmacol* 2003, **3:**747-756.

24. Rappeneau S, Baeza-Squiban A, Jeulin C, Marano F: **Protection from cytotoxic effects induced by the nitrogen mustard mechlorethamine on human bronchial epithelial cells in vitro.** *Toxicol Sci* 2000, **54:**212-221.
25. Das SK, Mukherjee S, Smith MG, Chatterjee D: **Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue.** *J Biochem Mol Toxicol* 2003, **17:**177-184.
26. Ying W, Chen Y, Alano CC, Swanson RA: **Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes.** *J Cereb Blood Flow Metab* 2002, **22:**774-779.
27. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB: **Alkylating DNA damage stimulates a regulated form of necrotic cell death.** *Genes Dev* 2004, **18:**1272-1282.
28. Ying W, Alano CC, Garnier P, Swanson RA: **NAD+ as a metabolic link between DNA damage and cell death.** *J Neurosci Res* 2005, **79:**216-223.

Appendices: